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Original Article

Preeclampsia is associated with low placental transthyretin levels

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ABSTRACT

Objective: To investigate the relationship between placental transthyretin (TTR) level and preeclampsia.
Materials and Methods: Placental tissues from uncomplicated and preeclamptic pregnancies were analyzed using immunohistochemistry and image analysis. We measured the mean optical density (OD) of immunohistochemical staining of TTR across multiple sections using Image Pro Plus 6.0. To avoid bias, we used placental tissue array, which contained preeclamptic placentas ($n = 8$) and the control placentas ($n = 6$) on the same slide.
Results: The mean TTR OD of the syncytiotrophoblast layer of placentas (95% confidence interval) from the first trimester was higher than those from the second/third trimester, and term placentas [0.149 (0.014–0.285) for the 1st trimester, 0.037 (0.000–0.073) for the 2nd/3rd trimester, and 0.011 (0.035 –0.056) for term; $p < 0.01$]. Although the OD of the second/third trimester placentas appeared greater than that of term placentas, this was not statistically significant. The mean TTR OD of the syncytiotrophoblast layer of the severe preeclampsia group was lower than that of controls [0.010 (0.005–0.016) vs. 0.027 (0.013–0.041), $p < 0.05$].
Conclusion: The immunohistochemical expression of TTR in the syncytiotrophoblast layer of the placenta decreased significantly after 12 weeks of gestation, paralleling the changing demands of thyroid hormone uptake into the placenta. The reduced TTR expression in the syncytiotrophoblast layer of the preeclamptic placenta might impair thyroid hormone uptake and contribute to the pathophysiology of the disease.

Introduction

Preeclampsia (PE) is an idiopathic multisystem disorder characterized by new-onset hypertension and proteinuria after 20 weeks of gestation. It is the leading cause of maternal and perinatal morbidity and mortality [1]. The pathophysiology underlying PE is still incompletely understood, although it is widely accepted to involve a failure of appropriate placental development [2]. Differential gene and protein expression [3], or initial low oxygen environment [2] can cause abnormal growth of placenta and fetus [4]. Nevertheless, the precise cause of the pathological changes within the placenta prior to the onset of PE is still unclear.

The critical role of thyroid hormones (THs) in the development of many organs is well recognized. Hypothyroidism may cause infertility, increased risk of miscarriage, and other obstetric complications including PE [5,6]. Transthyretin (TTR), also known as prealbumin, is a TH carrier, and it has been suggested that TTR might influence the proliferation and differentiation of placental trophoblast through regulating exposure of placental cells to TH [7]. As a carrier protein, TTR transports multiple molecules, including THs and retinol. Elevated levels of TTR have been reported in the amniotic fluid of fetuses with abnormal karyotypes, including trisomy 21 and trisomy 18 [8]. In serum, TTR levels decrease after 12 weeks of gestation [9], similar to the changing demands of TH of fetus. In the placenta, TTR seems to be constant after 13 weeks of gestation [10]; however, the increased expression of TTR in the matrix and vessels within the placenta after 13 weeks suggests that

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expression in the syncytiotrophoblast (where the TTR transporter regulates TH uptake) should be reduced then.

Several studies compared total TTR levels between severe preeclampsia (sPE) and control placentas and found no correlation [11,12]. Recognizing TTR's role as a placental transporter, we aimed to study its spatial distribution. In this study, we measured TTR expression and distribution in normal placentas across gestation. We also compared TTR expression in sPE placentas and gestational matched controls to investigate the possible relationship between PE and placenta TTR.

Materials and methods

Patients

The diagnosis of sPE was based on the criteria of the National High Blood Pressure Education Program Working Group on high blood pressure in pregnancy. The criteria included the presence of high blood pressure of $\geq$ 160/110 mmHg on two occasions at least 6 hours apart that occurred after 20 weeks of gestation in women with previously normal blood pressure, accompanied with proteinuria ($\geq$ 5 g/24 hours) or proteinuria of $\geq$ 3+ on two random urine samples collected at least 4 hours apart. None of the patients had a history of hypertension or renal diseases. The control participants were age matched without hypertension or proteinuria or other pregnancy complications except preterm labor. This study was conducted in accordance with the provisions of the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Research Centre for Women's and Infant's Health (RCWIH) Biobank at Mount Sinai Hospital (Mount Sinai Hospital Research Ethics Board, MSH REB# 04-0018-U). Written informed consent was obtained from all participants.

We examined TTR expression in normal placentas across gestation. First trimester placentas (6–12 gestational weeks) were obtained from elective terminations of pregnancies by dilatation and curettage. Second trimester and preterm placentas were from elective termination and from preterm labor patients without other pregnancy complications. Term placentas were from spontaneous labor or cesarean delivery.

Placenta immunohistochemistry

All samples were subjected to the same experimental methods. Briefly, paraffin blocks were cut into 4-μm-thick sections. After dewaxing in xylene and rehydration in graded alcohol, the sections were heated in 0.1 mol/L sodium citrate buffer (pH 6.0) in a microwave oven for 10 minutes for antigen retrieval. The slides were then incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Nonspecific binding was blocked by incubation in blocking solution (protein block, serum-free solution; Dako, Carpinteria, CA, USA) for 10 minutes. Subsequently, the slides were incubated for 1 hour at room temperature with specific Anti-Human-Transthyretin antibody (Dako) diluted 1:200; in the case of the negative controls, the primary antibody was replaced with phosphate-buffered saline. Slides were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (Dako; 1:300 dilution) for 30 minutes at room temperature. Substrate-chromogen DAB was added to each slice for 2 minutes, and the reaction was stopped by washing in excess tap water. Hematoxylin was used as a counterstain.

Western blotting

Placental tissue from 10 sPE patients and six gestational age-matched controls was homogenized in 250mM sucrose, 10mM Hepes/Tris, pH 7.4 buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Next, 40 μg total protein was separated in NuPAGE Novex 4–12% Bis/Tris gradient gels (Invitrogen, Grand Island, NY, USA) and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA) for 30 minutes. Blotting was performed by incubation of the membranes with Rabbit anti-TTR (Dako) antibody (1:1000 dilution) overnight at 4°C. Membranes were incubated with the secondary antibody (antirabbit immunoglobulin G-HRP; 1:3000 in Tris-buffered saline/Tween 20; Dako) for 30 minutes. Detection of bound antibody was performed with Pierce chemiluminescent (ECL) kit (Thermo, Waltham, MA, USA). Levels of proteins were then quantified using Quantity-One program (Bio-Rad Laboratories). TTR protein levels were normalized to β-actin.

Placental tissue array

Immunohistochemical (IHC) technique is a qualitative examination, and its quantitative analysis might be influenced by many procedures including tissue selection, paraffin blocks section, and staining. To avoid these biases as much as possible, we used the method of placental tissue array. Tissue array slides were obtained from the RCWIH BioBank program of Mount Sinai Hospital, Toronto, Ontario, Canada, in accordance with the policies of the Mount Sinai Hospital Research Ethics Board (MSH, 10-0128-E). Each of the tissue array slides contains sections from eight placentas from sPE patients and six gestational matched preterm labor controls (Table 1). The placentas used for tissue array were chosen by an experienced pathologist, and each placenta was sampled from four separate locations. The tissue array makes it possible to test TTR in all the placentas at the same time using exactly the same IHC procedures. All tissues are located randomly on the slides and coded until the quantitative analysis was completed.

There was no significant difference in gestational age, gravidity, and parity between the sPE group and the control group. For all the placentas, no fetal growth restriction was diagnosed.

Quantitative analysis

TTR is expressed in both the syncytiotrophoblast layer and the stroma of the placenta. We used Image Pro Plus version 6.0 (Media Cybernetics, Bethesda, Maryland, USA) to analyze the digital images. This method of IHC quantification has been used by several other groups [13,14]. To avoid possible bias in light intensity during image capture and to ensure the accuracy of measurements, image brightness was normalized to background levels. All images are taken at exactly the same conditions using the same microscope and camera (Olympus DP72, ISO 200, Olympus, Tokyo, Japan). We selected syncytiotrophoblast layer and stroma separately as area of interest (AOI) for measurements. Using Image Pro Plus, we changed the images to grayscale digital images to test total optical density (OD) and calculate the area of AOI. We then calculated the mean OD using total OD/area of AOI. The mean OD represents the density of dye staining and reflects the content of TTR.

Table 1
Characteristics of the severe PE and control groups.

	sPE	Controls
n	8	6
Gestational age (wk)	29 ± 1.7	31 ± 2.4
Gravidity	1.4 ± 0.5	2.0 ± 1.1
Parity	1.0 ± 0.0	1.5 ± 0.8
Placental weight (g)	281 ± 60	385 ± 91
Birth weight (g)	1046 ± 238	1822 ± 328

sPE = severe preeclampsia.

Statistical analysis

Data were transported into SPSS version 13.0 (SPSS Inc., Chicago, IL, USA), and statistical analysis was performed using *t* test and one-way analysis of variance. A p value < 0.05 was considered statistically significant. Data were expressed as mean ± standard deviation. Graphic representation of the data was prepared using GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA, USA).

Results

Placenta immunohistochemistry

Six first trimester placentas (6–12 weeks of gestation), nine second/third trimester placentas (12–37 weeks of gestation), and nine term (37–41 weeks of gestation) placentas were studied. Within first trimester placentas, syncytiotrophoblast cells showed strong positive staining for TTR whereas there was no staining in cytotrophoblast cells and stroma. In second/third trimester placentas, TTR staining in syncytiotrophoblast cells became less positive whereas the stroma became increasingly positive. In term placentas, TTR staining in syncytiotrophoblast cells was very weak, whereas endothelial cells, some blood cells, and some stromal cells become even more positive than second trimester placentas. Thus, it appears that TTR levels in syncytiotrophoblast cells decrease with the increasing gestational age whereas levels in the stroma increase (Figure 1).

Quantitative analysis was conducted using Image Pro Plus to calculate the mean OD of TTR staining. Initial analysis was conducted using the entire placental area (trophoblast layers and stroma) and showed no differences in TTR levels across gestation. However, when the analysis was conducted specifically on the syncytiotrophoblast layer, the mean OD of first trimester, second/third trimester, and term placentas was 0.149 [95% confidence interval (CI), 0.014–0.285], 0.037 (95% CI, 0.000–0.073), and 0.011 (95% CI, 0.035–0.056), respectively (Figure 1). Analysis of variance revealed that TTR staining of first trimester placenta was much stronger than that of second/third trimester ($p < 0.01$) and term placenta ($p < 0.01$). Although the OD of second/third trimester placenta appeared greater than the OD of term placenta, this observation did not reach statistical significance.

Western blotting

TTR is detected in both sPE and age-matched placentas as a single band at approximately 16kDa, representing the TTR monomer under dissociating condition. There was no difference in the TTR expression of sPE placentas and controls (Figure 2).

Placenta tissue array

The tissue array slides included placentas from eight PE patients and six gestational age-matched controls. Each placenta is sampled from four different locations. After staining, photographs of four random fields for each placental section were taken, and the mean OD of the 56 photographs was calculated. All photographs were coded before the quantitative analysis was conducted. As with the routine placental sections described above, when analysis of the sections included all placental layers (trophoblast and stroma), there was no difference in TTR expression in sPE and control groups (Figure 3). But when the syncytiotrophoblast layer was selected for analysis, the mean OD of the sPE group was significantly lower than that of the control group [0.010 (95% CI, 0.005–0.016) vs. 0.027 (95% CI, 0.013–0.041); $p < 0.05$].

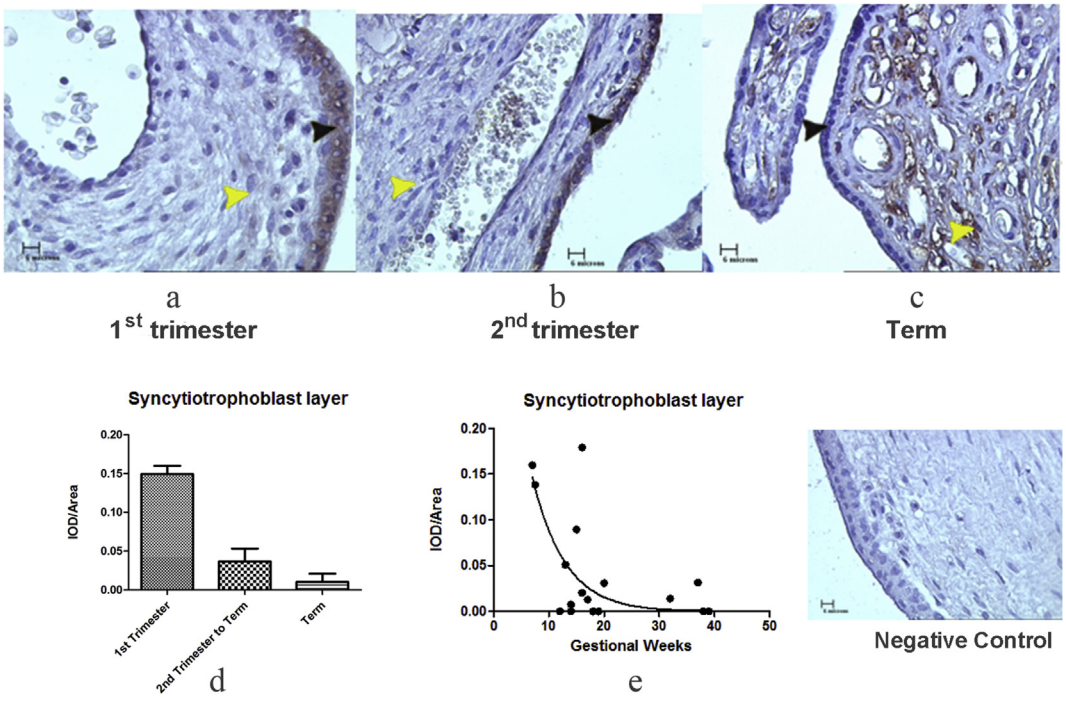

Figure 1. Transthyretin (TTR) expression in placenta across gestation. Black arrows identify syncytiotrophoblast layers and yellow arrows indicate stroma of placentas. Scale bars = 6 μm. Syncytiotrophoblast TTR staining becomes progressively weaker with advancing gestation. TTR staining in the stroma shows an inverse relationship to that of the syncytiotrophoblast. (A) Syncytiotrophoblast cells stain strongly positive for TTR in first trimester placenta. (B) During second trimester, syncytiotrophoblast TTR became weaker, but stroma TTR started to express. (C) Till term, syncytiotrophoblast TTR was quite weak, but stroma TTR became strong. (D) Quantitative analysis revealed that TTR staining of syncytiotrophoblast layer of first trimester placenta was much stronger than second/third trimester ($p < 0.01$) and term placenta ($p < 0.01$). Although the optical density (OD) of second/third trimester placenta appeared greater than term placenta, this was not statistically significant. (E) Syncytiotrophoblast TTR staining becomes progressively weaker with advancing gestation.

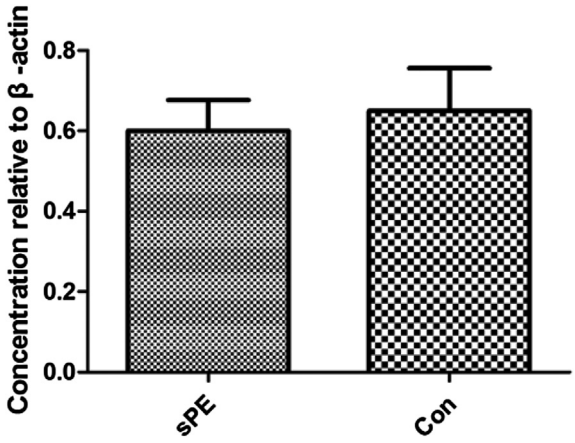

Figure 2. When we directly compared total transthyretin (TTR) in placentas using Western blotting test, no significant difference was found between severe preeclampsia (sPE) placentas and controls.

support the fetal development [15]. Even after 12 weeks of gestation, the fetus still requires additional transport of THs from the mother [15].

The functional form of TH is T3. The availability of the active ligand T3 within tissues is locally determined by the action of the iodothyronine deiodinases. There are three subtypes—D1, D2, and D3. T3 is generated by the activity of D1 and D2, via 5# reductive or outer ring deiodination of the prohormone T4 to 3,5,3′-triiodothyronine, whereas D3 converts T4 to 3,3,5′-triiodothyronine (reverse T3, rT3), acting as a deactivating enzyme for THs [16]. In placenta, there is only D2 and D3. T4 is transported into cells, and deiodinated by D2, and converted to T3 [16].

T3 can act directly on placental tissue and influence the proliferation and differentiation of trophoblast cells. It enhances the production of epidermal growth factor (EGF) by trophoblast cells *in vitro* [17,18], and the expression of integrins and matrix metalloproteinases (MMPs) in cultured early placental extravillous trophoblasts (EVTs) [18].

The placenta has high levels of D3, which acts as a barrier to TH uptake. TH thus requires active transporters to enter the

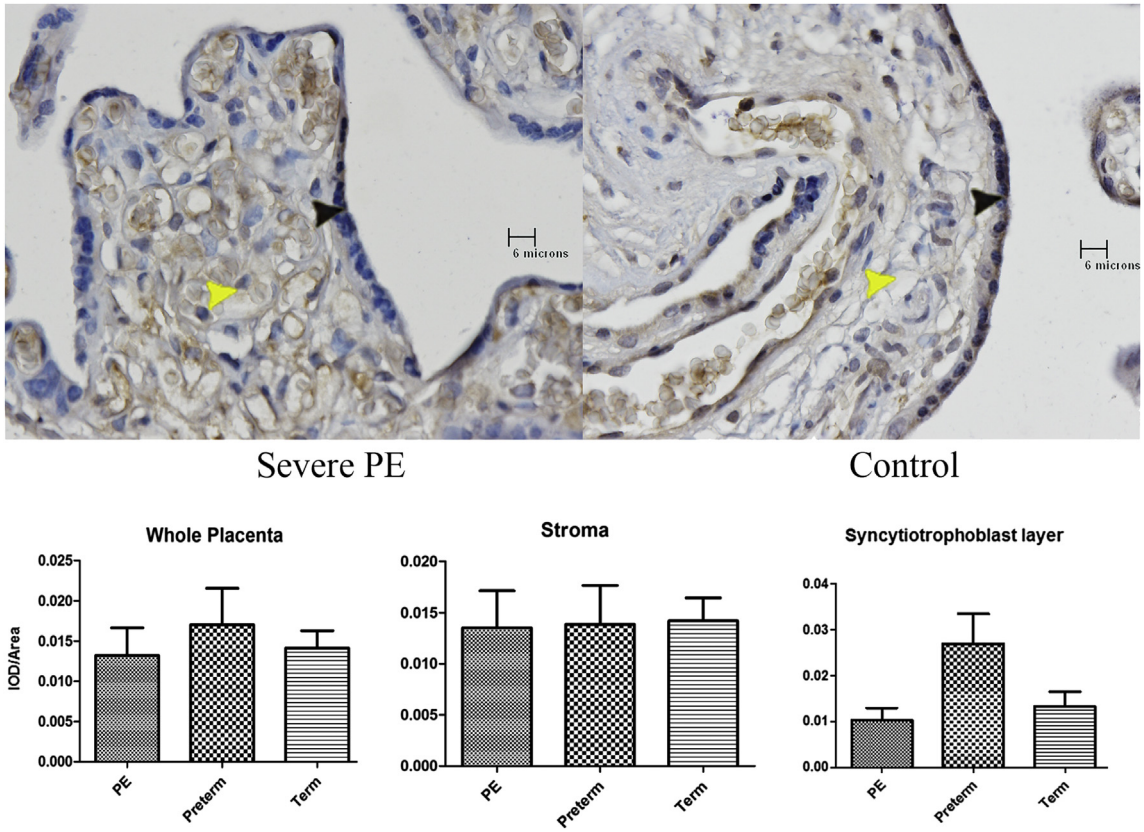

Figure 3. Transthyretin (TTR) expression in severe preeclampsia (sPE) placentas and controls. Black arrows identify syncytiotrophoblast layers and yellow arrows indicate stroma of placentas. Scale bars = 6 μm. TTR expression in whole placenta or stroma of sPE patients was not significantly different from gestational matched controls, but in syncytiotrophoblast it was significantly reduced compared with controls ($p < 0.05$).

Discussion

PE causes millions of maternal and perinatal deaths every year; however, the mechanisms underlying its pathophysiology remain unclear. Our research focused on the TH carrier, TTR.

THs are very important for growth and differentiation of many organs. Human fetal TH synthesis is initiated at about 12 weeks of gestation, and thus in the first trimester TH needs to be transported from the maternal circulation across the placental barrier to placenta [7,19,20]. The precise mechanism by which T4 is transported into trophoblast cells is not clear. Certain proteins, such as members of organic anion-transporting polypeptides, monocarboxylate transporter, can transport T4 or T3 across the membrane [21]. TTR plays an important role in TH transportation [7,22]. Human placenta trophoblast cells can secrete and internalize TTR, and the internalization is dramatically increased in the presence of T4 (^{125}I-T4) or T3, which enter cells as a complex with TTR [22].

Our results showed that in normal placentas, TTR staining is most positive in syncytiotrophoblast cells from the first trimester and decreases with gestational age until term when expression is very weak or negative. Within the stroma, however, staining appeared in the second trimester and became stronger with increasing gestational age. These observations correlate with the fetal changing demands for TH transport across the placenta. Once the fetal thyroid gland begins to produce TH, additional TH transport from the maternal circulation could harm fetal growth and development—consequently, TTR levels in the trophoblast are decreased and further reduced till term when the fetal thyroid can produce all of the required THs. This result differs somewhat from the study of Patel et al [10], who found that TTR levels increased to the end of the first trimester then remained high until term. This apparent discrepancy might be attributed to the differential expression of TTR between the syncytiotrophoblast layer and the stroma. In the context of a placental transporter, it is more informative to study TTR expression in the syncytiotrophoblast layer.

The changing level of TTR expression in the syncytiotrophoblast across gestation is intriguing. TH can influence trophoblast proliferation and differentiation as well as contribute to increased placental trophoblast invasiveness [17,18,23]. The ability of TTR to aid transport of TH across the placental barrier [7,22] suggests that this protein may play an important role in normal placental development especially in the first trimester. In a previous study using proteomic methodology, we found that the TTR serum levels are greatly reduced in PE patients [24]. Our current study suggests that TTR expression by syncytiotrophoblast cells might be a contributing pathway to PE. Our initial examination using Western blotting showed no difference in TTR levels in the placenta of sPE patients compared to preterm controls. This is consistent with other proteomic studies that found no significant difference [11] or even upregulation of TTR [12] in placentas from PE patients. We questioned whether this might be attributed to TTR synthesized by other placental cell types or to the fetal circulation entering the placenta stroma. We therefore investigated the differential expression of TTR between the syncytiotrophoblast and the stroma. Our results confirmed that TTR levels are significantly reduced in the syncytiotrophoblast of sPE placentas as compared to age-matched controls. The total placental expression of TTR (i.e., when stromal expression is included) shows no difference between PE and age-matched controls. These findings likely explain why analysis of TTR from whole placentas failed to detect differences in expression in PE and controls.

In summary, we have documented inverse changes in the levels of TTR in syncytiotrophoblast and stromal compartments of the placenta across gestation. We have also shown that syncytiotrophoblast TTR levels, but not the stromal expression, are reduced in patients with PE compared to patients without PE at a similar gestational age (preterm birth). The fact that these changes are only detected when cell layer-specific analyses are conducted may account for the failure of previous studies to detect altered placental TTR levels in association with PE. The consequences of the reduced level of TTR in the syncytiotrophoblast remains to be determined, but we speculate that reduced TH transport in the first trimester would impair trophoblast proliferation and differentiation and that the compromised placental development might contribute to the pathogenesis of PE.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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